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Determinants of selectivity in Xer site-specific recombination

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A remarkable property of some DNA-binding proteins that can interact with and pair distant DNA segments is that they mediate their biological function only when their binding sites are arranged in a specific configuration. Xer site-specific recombination at natural plasmid recombination sites (e.g., *cer* in ColE1) is preferentially intramolecular, converting dimers to monomers. In contrast, Xer recombination at the *Escherichia coli* chromosomal site *dif* can occur intermolecularly and intramolecularly. Recombination at both types of site requires the cooperative interactions of two related recombinases, XerC and XerD, with a 30-bp recombination core site. The *dif* core site is sufficient for recombination when XerC and XerD are present, whereas recombination at plasmid sites requires ~200 bp of adjacent accessory sequences and accessory proteins. These accessory factors ensure that recombination is intramolecular. Here we use a model system to show that selectivity for intramolecular recombination, and the consequent requirement for accessory factors, can arise by increasing the spacing between XerC- and XerD-binding sites from 6 to 8 bp. This reduces the affinity of the recombinases for the core site and changes the geometry of the recombinase/DNA complex. These changes are correlated with altered interactions of the recombinases with the core site and a reduced efficiency of XerC-mediated cleavage. We propose that the accessory sequences and proteins compensate for these changes and provide a nucleoprotein structure of fixed geometry that can only form and function effectively on circular molecules containing directly repeated sites.

[*Key Words*: Site specific recombination; Xer; selectivity; recombinase]

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Site-specific recombination systems function in a range of programmed DNA rearrangements in microbes that include helping to specify developmental pathways in bacteria and bacteriophages (Sato et al. 1990; Landy 1993; Carrasco et al. 1994); determining cell type and virus host range (Zieg and Simon 1980; Hiestand-Nauer and Iida 1983; Klemm 1986; Klippel et al. 1988; Tominaga et al. 1991); processing the products of genetic transposition (Arthur and Sherratt 1979); and controling circular replicon copy number and inheritance (Summers and Sherratt 1984; Futcher 1986; Blakely et al. 1991).

Odd numbers of homologous recombination events between circular replicons during or after replication, produces dimers that need to be converted to monomers before they can be segregated normally at cell division (Austin et al. 1981; Blakely et al. 1991; Kuempel et al. 1991). Plasmid dimers can also arise as a consequence of rolling circle replication during conjugal transfer (Warren and Clark 1980; Erickson and Meyer 1993). The Xer site-specific recombination system, initially discovered by its role in plasmid ColE1 stable inheritance, also functions in the normal inheritance of the *Escherichia coli* chromosome and the stable inheritance of other multicopy plasmids. Xer site-specific recombination uses two related recombinases XerC and XerD; these share 37% identity and belong to the lambda integrase family of site-specific recombinases (Blakely et al. 1993). XerC cleaves the "top" strand and XerD the "bottom" strand in in vitro recombination assays (Fig. 1A; Arciszewska and Sherratt 1995; Colloms et al. 1996; Sherratt et al. 1995; G. Blakely and D.J. Sherratt, unpubl.). Mutations in XerC, XerD, or in the chromosomal recombination site *dif* produce a subpopulation of cells that are filamentous and contain aberrant nucleoids. This phenotype is suppressed by mutations that abolish homologous recombination, supporting the idea that Xer recombination "undoes" the "damage" caused by homologous recombination (Blakely et al. 1991; Kuempel et al. 1991; Leslie and Sherratt 1995).

Integrase family recombinases contain four highly conserved amino acids, the RHRY tetrad (Argos et al. 1986; Abremski and Hoess 1992). The tyrosine nucleophile attacks the phosphodiester bond to be cleaved, generating a 3′ phosphotyrosyl protein and a free 5′ hydroxyl end (Pargellis et al. 1988). The other conserved residues have been implicated in activation of the phosphodiester bonds before nucleophilic attack and religation of cleaved strands (Chen et al. 1992; Pan and Sadowski 1992). Site-specific recombination mechanisms are reviewed by Stark et al. (1992).

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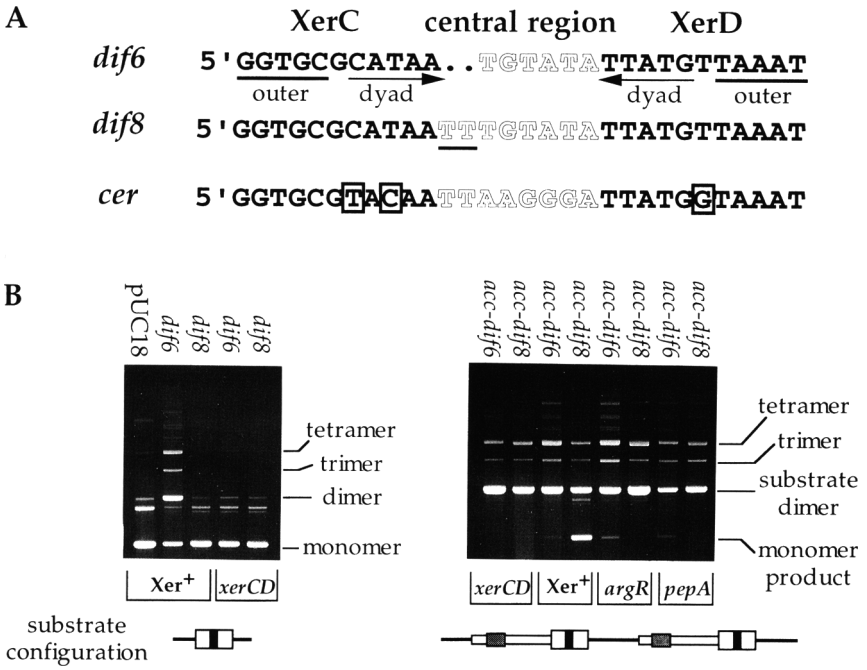

Figure 1. (*A*) Sequence comparison of Xer core recombination sites: *dif6*, from the *E. coli* chromosome; *dif8*, containing an additional TT dinucleotide (underlined) in the central region; *cer*, from ColE1. The base differences in left and right half-sites between *dif6* and *cer* are boxed. The putative outer recognition sequences are underlined, and arrows indicate the region of dyad symmetry in each half-site. (*B*) Analysis of in vivo multimerization for plasmids containing *dif6* or *dif8* after transformation of monomers into Xer$^+$ or *xerCD* strains (*left*); *dif6* was a substrate for Xer-mediated intermolecular recombination as indicated by presence of multimers; *dif8* containing plasmids remained as monomers in the Xer$^+$ strain. Intramolecular resolution of plasmid dimers containing *acc–dif8* to monomer product (~30% of total DNA) only occurred in the Xer$^+$ strain (*right*), demonstrating its dependence on the accessory proteins ArgR and PepA. Resolution and multimerization of plasmid dimers containing *acc–dif6* was independent of accessory proteins. Note that the supercoiled tetramer band may contain some nicked dimer. Trimer formation in Xer$^-$ strains probably occur by homologous recombination within tetramers.

A specific 33-bp DNA fragment contains a functional *dif* site as assayed by normal chromosomal segregation and by its ability to promote Xer recombination when inserted into a plasmid (Blakely et al. 1991). *dif* consists of an 11-bp XerC-binding site separated by a 6-bp central region from a partially dyad symmetrical 11-bp XerD-binding site (Fig. 1A; subsequently we refer to this wild-type *dif* core site as *dif6*). The interaction of the recombinases at *dif6* is highly cooperative and can lead to both intermolecular and intramolecular recombination when the site is present in a plasmid.

In contrast, recombination at natural plasmid sites (e.g., *cer* in ColE1 and *psi* in pSC101) is preferentially intramolecular, converting dimers (and higher multimers) to monomers. This selectivity for intramolecular resolution is correlated with a requirement for "accessory" proteins in addition to the recombinases and ≤200 bp of accessory sequences adjacent to the 30-bp recombination core site (Summers and Sherratt 1984; Summers 1989; Sherratt et al. 1995). Absence of *cer* accessory sequences or the accessory proteins ArgR and PepA makes the *cer* core site inactive in recombination (Stirling et al. 1988, 1989). What determines whether recombination will be preferentially intramolecular and require accessory factors, or both intermolecular and intramolecular, requiring only recombinases and a recombination core site? Summers (1989) demonstrated that two variant *cer* sites, obtained by recombination between *cer* and its plasmid CloDF13 homolog *parB* had very different properties. Both contained the *cer* XerC- and XerD-binding sites , but one (the "type I hybrid"), like *cer*, contained an 8-bp central region (although different in sequence to *cer*), whereas the other (the "type II hybrid"; here designated as *cer6*) was deleted for a central region TT dinucleotide adjacent to the XerC-binding site and had a 6-bp central region. The type I hybrid had the recombination properties of *cer*, requiring accessory sequences and proteins and recombining intramolecularly. In contrast, *cer6* could recombine both intermolecularly and intramolecularly when accessory proteins and sequences were absent, although it exhibited preferential intramolecular recombination when accessory sequences were present. This indicated that central region size differences could determine recombination requirements and outcomes. Nevertheless, other differences in core site sequence can influence the requirements and outcomes of recombination. For example, CloDF13 *parB* and pSC101 *psi* each have a 6-bp central region, yet recombine preferentially intramolecularly when accessory sequences and proteins are present (Cornet et al. 1994; Roberts 1994; Colloms et al. 1996).

To understand more about how XerC and XerD interactions at a recombination core site determines whether accessory factors will be required and whether recombination will be preferentially intramolecular, we have used a model system that compares recombinase interactions with *dif6* and a derivative of *dif* containing an 8-bp central region (*dif8*). These sites were chosen because they have a higher affinity for XerC than *cer* derivatives, thus facilitating analysis of recombinase bind-

ing. The differences in recombinase interactions with these sites provides a framework for understanding the requirements for accessory sequences and proteins in some sites and how this leads to selectivity for a particular recombination outcome.

Results

In vivo recombination properties of dif8, *a* dif *derivative containing an 8-bp central region.*

Wild-type *dif6*, when present in a plasmid, is a substrate for Xer-mediated intermolecular and intramolecular recombination, as assayed by the conversion of plasmid monomers to multimers in a Xer^+ strain that is deficient in plasmid homologous recombination (Blakely et al. 1991). A comparison of the ability of *dif6* and *dif8* (containing an additional TT dinucleotide inserted into the central region; Fig. 1A) to act as substrates for Xer-mediated intermolecular recombination is shown in Figure 1B. Although the *dif6*-containing plasmid was an efficient substrate for intermolecular recombination (~70% of the DNA being converted from monomers to multimers after ~40-cell generations). The *dif8*-containing plasmid produced the same levels of monomers (~90%) in Xer^+ and Xer^- strains. Therefore, *dif8* cannot function as an Xer recombination core site.

To determine whether *dif6* and *dif8* can respond to accessory sequences and proteins, derivatives were constructed that contained the *cer* accessory sequences upstream of the XerC-binding sites. We refer to these sites as *acc–dif6* and *acc–dif8*. To test these sites for intramolecular resolution, dimers of plasmids containing either *acc–dif6* or *acc–dif8* were used to transform Xer^+, *xerCD*, *argR*, and *pepA* strains and the resulting plasmid DNAs were analyzed. Whereas the *acc–dif6* dimers were substrates for both intermolecular and intramolecular Xer recombination that was independent of PepA and ArgR (Fig. 1B), *acc–dif8* only recombined intramolecularly in a reaction that requires PepA, ArgR, XerC, and XerD (Fig. 1B). This is similar to the behavior of *cer*, although note that resolution of *acc–dif8* dimers was incomplete (~30% of the DNA was monomeric after ~40 generations), compared to the ≥80% resolution of *cer* dimers in similar experiments (Summers and Sherratt 1984). This incomplete resolution could be a consequence of a 1-bp insertion between the core site and accessory sequences in *acc–dif8* compared to *cer* (see Materials and methods).

Taken together these results show that the addition of 2 bp between the recombinase binding sites of *dif6* destroys the site's ability to act as a core site for Xer-mediated recombination, both for intermolecular and intramolecular events. Recombination proficiency is only restored to *dif8* when accessory sequences and proteins are present; then resolution selectivity is imposed on the site. The addition of accessory sequences to *dif6* appeared to have little effect on its ability to recombine and did not impose directionality to its reactions. This contrasts to the properties of *cer6*, which shows resolution selectivity in the presence of ArgR and PepA, but recombines intermolecularly and intramolecularly in their absence (Summers 1989). Therefore, the sequence differences in the core sites of *cer* and *dif* may also play a role in determining whether a core site responds to accessory sequences and proteins.

Recombinase binding to dif6 *and* dif8

Because binding of XerC and XerD to *dif* is known to be highly cooperative (Blakely et al. 1993), we believe it likely that the proteins interact when bound to their target. The addition of an intervening 2 bp to B-form DNA will rotate the relative positions of the two recombinase binding sites by ~69° and separate them by 6.8Å. Such rotation and spatial separation may be expected to alter the ability of recombinases to interact across the intervening DNA (Mao et al. 1994).

Differences in cooperative binding of the recombinases to *dif6* and *dif8* were analyzed using gel retardation to measure the binding of XerC and XerD to *dif*-containing fragments as a function of recombinase concentration. The apparent equilibrium dissociation constant (K_d) for each of the recombinases binding individually to *dif6* and *dif8* were similar; ~177 nM for XerC binding and ~6 nM for XerD binding, indicating that the 2-bp insertion does not influence the binding of either recombinase alone and confirming our earlier observation that XerD binds with a higher affinity than XerC. Titration of XerC in the presence of constant XerD (250 nM) for the two sites indicated that the apparent K_d of XerC for a *dif6*/XerD complex was ~0.5 nM whereas the apparent K_d of XerC for a *dif8*/XerD complex was ~2.4 nM (Fig. 2A). This indicates a reduction in cooperativity index from ~300 fold for *dif6* to 60- to 75-fold for *dif8*, a reduction in overall affinity of ~5-fold.

Complexes of XerC and XerD with *dif8* migrate more slowly than complexes with *dif6* (see Fig. 2B), indicating that the *dif8*/XerC/XerD complex has undergone additional bending (Wu and Crothers 1984). This is similar to the increased mobility of a recombinase/*cer* complex relative to a recombinase/*dif* complex (Blakely et al. 1993). Because of this difference, we used circular permutation experiments to analyze the nature of potential alterations in bending induced by XerC and XerD. A bend in the center of a fragment will reduce its mobility in relation to the same bend at the end of the fragment (Wu and Crothers 1984; Lane et al. 1992).

XerC bound alone to either *dif6* or *dif8* induced a similar small bend [~25°; calculated using the method of Thompson and Landy (1988)]. XerD induced a larger bend in both sites (~40°). Measurement of relative mobilities of *dif6* and *dif8* complexes with XerC and XerD shows that the overall bend angle (note this is a composite angle because of bends induced by XerC and XerD) was greater for *dif8* (60°–65°) than for *dif6* (40°–45°). The fact that there may be more than one bend present precludes us from estimating a meaningful bend center.

We offer three explanations for the differences in overall bend angles for *dif6* and *dif8*: (1) the individual XerC

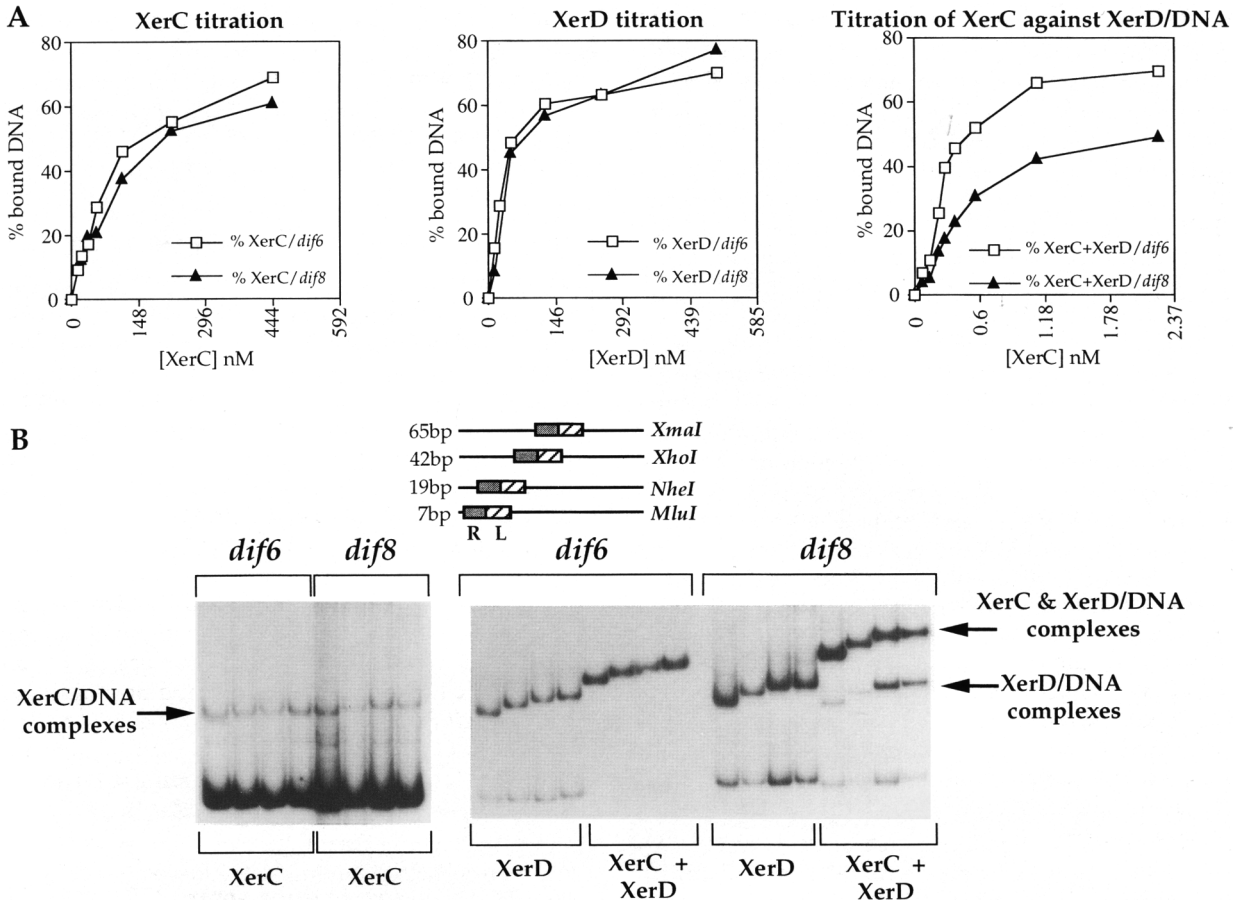

Figure 2. (*A*) Comparison of binding curves for titration of each recombinase with core recombination sites demonstrates similar apparent affinities for XerC and XerD binding to left and right half-sites, respectively, for both *dif6* and *dif8*. In the presence of XerD and higher concentrations of XerC (≥100 nM), >90% of DNA is bound. (*B*) Bending analysis using gel retardation for recombinases bound to DNA fragments containing either *dif6* or *dif8* generated by restriction enzyme digestion of plasmids based on pBEND2. Restriction fragments generated by *Mlu*I, *Nhe*I, *Xho*I, and *Xma*I, from left to right for each set of four reactions. Distances from the right half-site to the end of the DNA fragments are indicated. Note that the XerC/XerD/*dif8* complex is more retarded than the equivalent *dif6* complex, indicating an altered geometry.

and XerD bends in *dif6* and *dif8* remain the same, with the 69° relative rotation of binding sites in *dif8* producing a phasing change between bends induced individually by each recombinase; (2) an additional bend is introduced in the *dif8* DNA either as a direct consequence of the TT dinucleotide insertion, or because of changes in recombinase interactions induced by the spacing change; and (3) the individual bends induced by XerC and XerD are different in the two sites as a consequence of changes in DNA sequence or recombinase interactions. These explanations are not mutually exclusive and we cannot distinguish between them on the basis of these experiments. Altered geometry, leading to reduced electrophoretic mobility, appears to be diagnostic for recombination sites that show resolution selectivity.

In vitro XerC-mediated cleavage assay

The inability of *dif8* to undergo recombination, without the presence of accessory sequences, suggested that this site was defective in one or more of the following steps: synapsis, strand cleavage, and strand exchange. Strand cleavage can be assayed by the accumulation of recombinase–DNA covalent complexes in linear duplex "suicide" substrates that contain a nick three nucleotides 3′ from the recombinase-mediated cleavage site. Cleavage releases a trinucleotide, thus trapping the covalent protein–DNA complex because there is no adjacent 5′ OH that can attack the phosphotyrosine and reverse the reaction (Nunes-Duby et al. 1987; Sherratt et al. 1995). Suicide substrates based on *dif6* and *dif8* were used to compare the cleavage efficiences between the two sites and determine whether *dif8* is a substrate for XerC cleavage. A *cer* core site substrate was also used to ascertain the ability of XerC to cleave at another site that contained an 8-bp central region.

Defined concentrations of recombinases that ensured saturation of binding sites (confirmed by gel retardation; 1 μM each of XerC and XerD) were added to top strand suicide substrates containing a nick three nucleotides 3′

from the XerC cleavage site and the amount of covalent XerC–DNA complex determined by electrophoresis through polyacrylamide containing 0.1% SDS. Sixty to 70% of *dif6* DNA was converted to XerC covalent complexes in a 60-min reaction (Fig. 3). A weak second band running above the XerC/*dif6* covalent complex is possibly a strand transfer product resulting from a nucleophilic attack of a 5′ OH end of an oligonucleotide from a second covalent complex. Reactions containing an XerC–maltose-binding protein fusion confirm that top strand cleavages were mediated by XerC; note the greater retardation attributable to the size of the fusion protein. Half molecule products resulting from XerD-mediated bottom strand cleavage of *dif6* (~1% of total DNA) were also detectable as faint bands running below the substrate. The amount of covalent complex produced by XerC cleavage of *dif8* in a 60-min reaction was 2%–3% of total DNA, which was comparable to the amount of complex formed with the *cer* core sequence. Therefore, inefficient XerC cleavage of a core recombination site correlates with resolution selectivity and requirement for accessory factors. Note that the cleavage substrates used here may not assay every cleavage event; the trinucleotide generated by recombinase cleavage could remain bound to the duplex as a consequence of recombinase/DNA interactions and therefore, be available for reversal of the reaction. Nevertheless, we believe that comparative measurements of cleavage at *dif6* and *dif8* do indicate a real difference in cleavage efficiency.

Comparison of XerC covalent complexes formed with a *cer6* substrate and a derivative containing an additional TT dinucleotide at the 3′ end of the central region confirmed that it is the 8-bp central region that causes the reduction in detectable cleavages and not the addition of the TT bases adjacent to the XerC cleavage position (data not shown). We conclude that the ability of XerC to cleave DNA is strongly influenced by the interactions between XerC and XerD and the geometry of the recombinase–DNA complex.

*Altered protection of recombinase/*dif8 *complexes from attack by the chemical nuclease OP-Cu*

The 1, 10-phenanthroline–copper (OP-Cu) reagent is a chemical nuclease that interacts with DNA through the minor groove, where it initiates an oxidative attack on the C1-hydrogen of deoxyribose leading to cleavage of the DNA backbone (Sigman et al. 1991; Spassky et al. 1988). Resistance to cleavage is caused by blocking access to the DNA either as a result of protein steric hindrance or altered geometry that narrows the minor groove. Enhanced cleavage by OP-Cu results from altered DNA geometry that leads to a widened minor groove.

An OP-Cu footprint for the recombinases bound individually to *dif6* has shown that XerC binds to the left half-site and XerD binds to the right half-site (Blakely et al. 1993; see Fig. 1A). Binding of both recombinases to *dif6* results in almost complete occlusion of the OP-Cu reagent from the core site, giving protection of 28 bp (Fig. 4). Analogous footprinting reactions of recombinases bound at *dif8* demonstrated that although protection of the right half-site resembled *dif6*, almost all of the central region and the innermost AA dinucleotide of the left half-site had become susceptible to nuclease attack (Fig. 4). Cleavages were enhanced at deoxyribose groups corresponding to base positions 2 and 3 on the top strand and −1 and 1 on the bottom strand (Fig. 4); these posi-

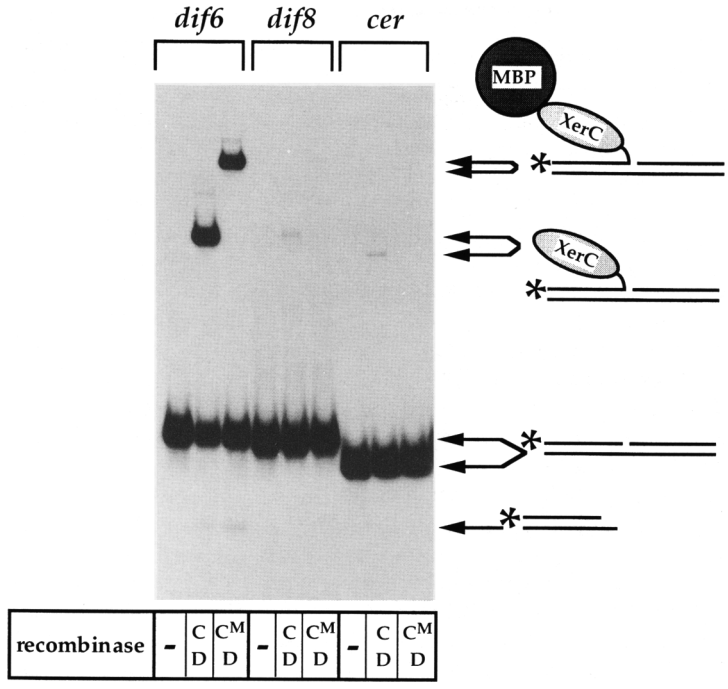

Figure 3. XerC-mediated cleavage at core recombination sites tested in vitro using radiolabeled (*) suicide substrates containing a nick in the top strand of the central region. Reactions (37°C; 60 min) containing XerC + XerD (CD) or XerC–maltose-binding protein + XerD (C^MD) were electrophoresed through 6% polyacrylamide containing 0.1% SDS and amount of substrate converted to covalent recombinase/DNA complex was quantified. The top strand of *dif6* was cleaved efficiently by XerC, whereas cleavage of top strands for both *dif8* and *cer* was substantially lower. Increased retardation of the recombinase/DNA complex in reactions containing XerC–MBP demonstrates that XerC was responsible for top strand cleavage. The half molecules visible below the substrate are generated by XerD cleavage of the bottom strand producing a double strand break.

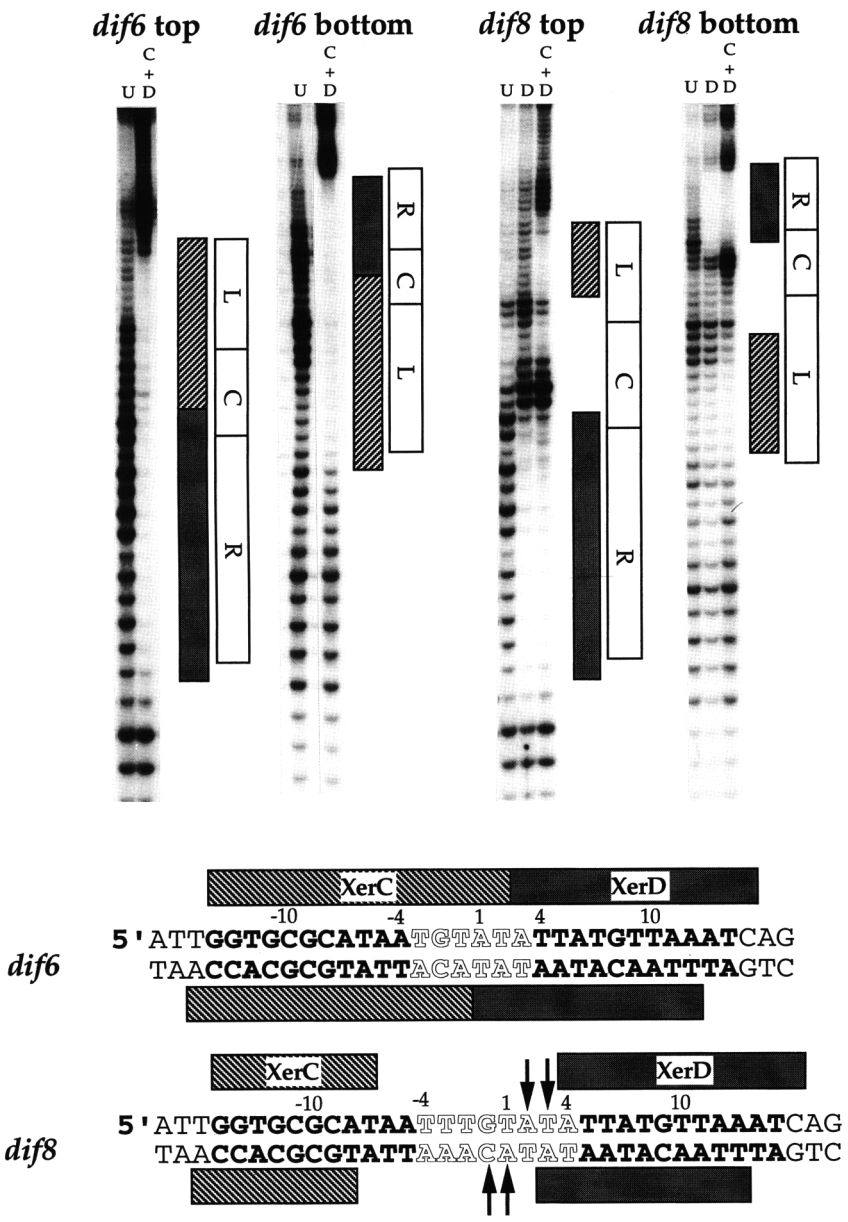

Figure 4. In situ 1,10-phenanthroline–copper footprinting was used to compare interactions of recombinases with *dif6* or *dif8*. Reactions containing XerC + XerD (C + D), XerD alone (D), or unbound DNA (U) are shown with regions protected from OP-Cu cleavage indicated as shaded boxes. Diagrammatic representations of core recombination sites are included adjacent to the autoradiographs and show left (L) and right (R) half-sites and central region (C). Sequences protected by XerC and XerD are shown at the *bottom*. Left and right half-sites are shown in boldface type, and the central region sequence is indicated as hollow lettering. Note the loss of protection for top and bottom strands of the dyad sequence in the left half-site and sensitivity of the central region in *dif8*. Arrows on the *dif8* sequence represent enhanced OP-Cu cleavages. Sequence coordinates are according to Blakely et al. (1993).

tions represent the symmetrical cleavages generated by the OP-Cu complex, where the nearest residue on the opposite strand is n + 2 across the minor groove (Conner et al. 1984). Binding of XerD alone to *dif6* (Blakely et al. 1993) and *dif8* (Fig. 4) also leads to some enhanced cleavage in the top strand central region adjacent to the right half-site. In contrast, there are no enhancements on the bottom strand in the presence of XerD alone. The altered patterns of protection and enhancements for the XerC/XerD/*dif8* complex are indicative of additional DNA distortions, which may contribute to the overall geometry of the complex as measured by electrophoresis. We also note that naked *dif6* and *dif8* DNA were not uniformly sensitive to OP-Cu cleavage; the central region and the dyad sequences were cleaved more readily than adjacent sequences (Fig. 4); we do not know if this is relevant to core site function.

The loss of protection for the innermost AA of the XerC-binding site, adjacent to where XerC cleaves (Fig. 4), suggests a reduced interaction of XerC with this region of the *dif8* DNA or a slight widening of the minor groove. This region of the XerC-binding site shows dyad symmetry with the inner region of the XerD-binding site and is presumably the recognition sequence for the catalytic domain of XerC. An altered disposition of XerC catalytic residues in relation to the strand cleavage position may explain the low levels of XerC-mediated cleavage observed with *dif8* suicide substrates.

Hydroxyl radical footprinting of dif6 *and* dif8

The small diffusible hydroxyl radical, which cleaves DNA with little or no sequence specificity, can be used to detect DNA deformation and protein binding (Tullius and Dombroski 1985, 1986). Gel retardation was used to separate recombinase-bound from unbound DNA after treatment with the reagents; footprints were only detected for XerD and not XerC when reactions were performed without gel retardation (data not shown); presumably because XerC binding is weak compared to XerD binding (this study; Blakely et al. 1993; Blakely and Sherratt 1994).

Large regions of protection from hydroxyl radical cleavage were detected on top and bottom strands for both *dif6* and *dif8*; there were no regions of strongly enhanced cleavage. Most of the central region of *dif6* was protected from cleavage (Fig. 5), again suggesting that the two recombinases come into close contact across the central region of this site. In contrast, the middle of the *dif8* central region is sensitive to hydroxyl radicals, further supporting the view of reduced recombinase contact in this region of *dif8*. The regions of dyad symmetry flanking the central region were protected on both strands, although the *dif8* XerC-binding site inner region was less protected than that of *dif6*, reinforcing the other evidence for reduced contact of XerC with this region of *dif8* (Fig. 5).

Protection of the three outer nucleotides of the XerC (top strand) and XerD (bottom strand) binding sites de-

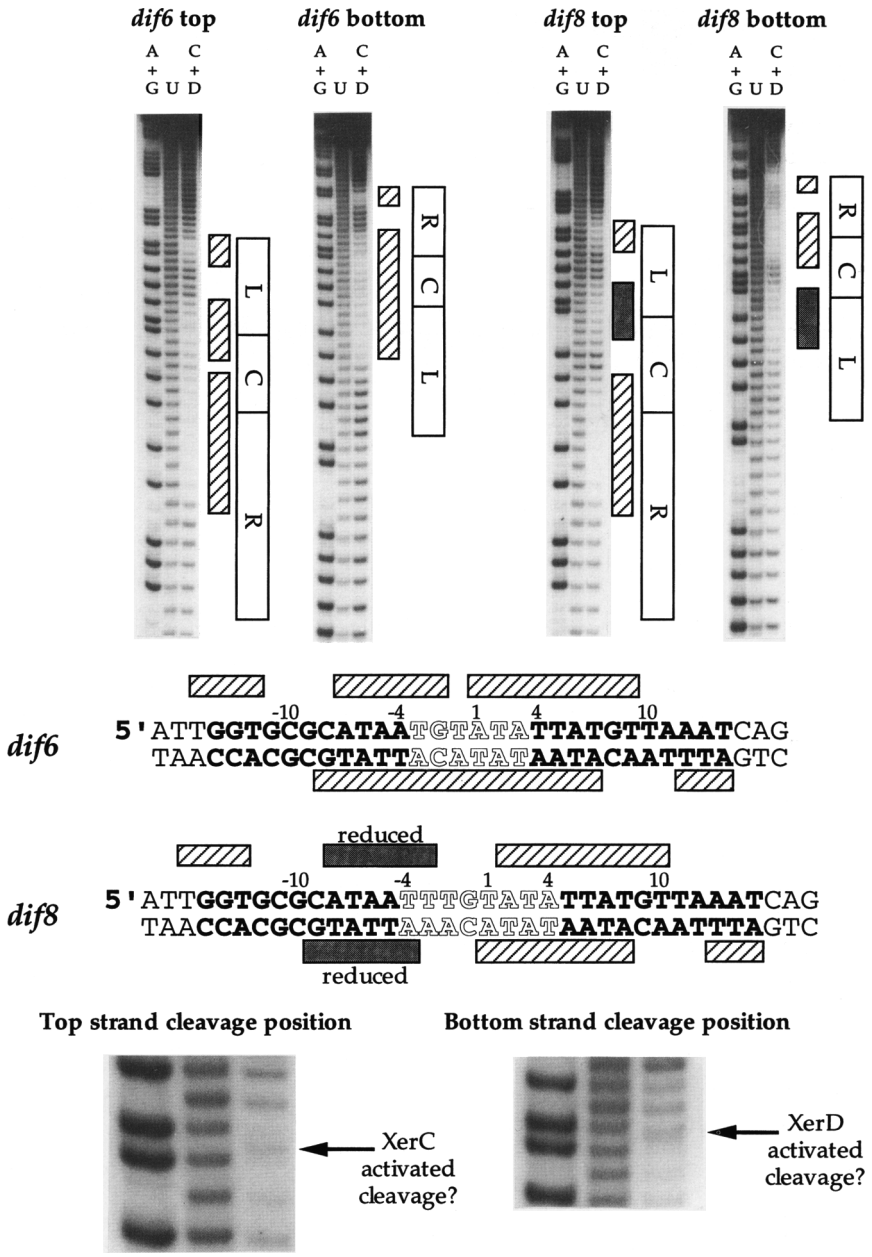

Figure 5. Hydroxyl radical footprints of XerC + XerD bound to *dif6* and *dif8*. Regions of core sites protected from cleavage in the presence of XerC + XerD (C + D), compared to unbound DNA (U), are indicated adjacent to the autoradiographs as are the relative positions of the core sites. Protection of outer sequences on both *dif6* and *dif8* denote contacts that may be involved in recognition specificity. Note reduced protection of the dyad portion of the left half-site sequence on both top and bottom strands, which is again indicative of reduced XerC interactions. The insets at the bottom of the figure illustrate that H_2O_2 can act as a nucleophile to mediate cleavage when a specific phosphodiester is activated by XerC and XerD. These cleaved products only occur when *dif6* is bound by XerC + XerD. Maxam-Gilbert A + G sequencing reactions (A + G) are included for each set of footprints.

lineate the outermost backbone contacts of the recombinases. These nucleotides along with the two immediately inside them have been implicated in contacts with XerC and XerD and in providing binding specificity (Blakely and Sherratt 1994). The 5′ TTT sequence in the bottom strand of the right half-site may be contacted by XerD through the major groove, as suggested by $KMnO_4$ interference footprinting (Blakely and Sherratt 1994). The hydroxyl radical protection pattern for the right half-site also shows similarity to the ethylation interference footprint for XerD binding (Blakely and Sherratt 1994), demonstrating the extensive interactions of XerD with the DNA backbone.

An additional weak band was observed in both top and bottom strand footprints (Fig. 5, insets). We believe that these correspond to cleavage mediated by H_2O_2 as the nucleophile and activated by XerC (top strand) and XerD (bottom strand); the 3′ end-labeled products should have 5′ OH groups, explaining why migration is retarded by approximately half a base pair on these sequencing gels (Blakely and Sherratt 1994). Kimball et al. (1993) reported that hydrogen peroxide can act as an exogenous nucleophile for cleavage of FRT DNA in the presence of FLP recombinase.

Discussion

Communication between distant DNA segments is important in a variety of biological reactions that include control of transcription, homologous recombination and gene conversion, mismatch repair, and site-specific recombination. In many of these interactions it is important for normal biological function that "correct interactions" occur to the exclusion of "incorrect interactions." For example, in activation of transcription, a gene's enhancers and bound transcription factors must activate expression of that gene but not that of neighboring genes (discussed in Corces 1995). Similarly, site-specific recombination can be limited to a pair of identical recombination sites in a particular configuration (discussed in Stark and Boocock 1995). A novel and intriguing feature of the Xer site-specific recombination system is that it can function with or without selectivity for a particular configuration of recombination sites, depending on the structure of the recombination site. This dual behavior is a valuable resource for analyzing the selectivity mechanism, as well as for providing insight into how selectivity can arise.

On all natural plasmid substrates for Xer recombination tested, recombination is preferentially intramolecular, converting multimers (that can arise by homologous recombination or by rolling circle conjugal transfer) to monomers. We believe that this resolution selectivity results from the use of a "topological filter" that ensures that productive synaptic complexes, which must have a fixed protein/DNA geometry, can only form and recombine on supercoiled substrates containing two directly repeated recombination sites (discussed in Boocock et al. 1987; Stark et al. 1989; Stark and Boocock 1995; Colloms et al. 1996). It is difficult to see how such a topological filter could operate to differentiate *dif6* sites on two separate monomeric chromosomes from two *dif* sites separated by 4.7 Mb on a dimeric chromosome. It is not surprising, therefore, to find that recombination at *dif6* does not show resolution selectivity, at least in the plasmid assays that we have available.

In the model system used here, a 2-bp increase in central region spacing from *dif6* to *dif8*, converts a core site that recombines intermolecularly and intramolecularly in vivo to one that shows preferential intramolecular resolution and requires adjacent accessory sequences and proteins. This mirrors precisely the in vivo recombination differences between *dif6* and the natural plasmid sites. The different in vivo recombination properties of *dif6* and *dif8* are correlated with changes in recombinase interactions with their targets, the geometry of the recombinase/core site complex, and the efficiency of XerC-mediated cleavage of DNA.

The simplest hypothesis that we can offer to explain the in vivo recombination properties of *dif6* and *dif8* is that a *dif6*/recombinase complex has the geometry and stability to be able to form a recombinational synapse with an identical partner complex using recombinase–recombinase interactions. Such synaptic structures may form irrespective of the configuration of the two sites, thus permitting intramolecular and intermolecular recombination. In *dif8*, as a consequence of changed core site/recombinase geometry and altered recombinase binding, catalytically active synapsed recombinase/core site complexes may be unable to form on any configuration of two sites. The addition of accessory sequences and the provision of accessory proteins that interact with these sequences could provide extra nucleoprotein "glue" that allows the formation of a catalytically proficient synaptic complex. We would expect this complex to have a fixed local geometry; this dictates that it can only form and function on directly repeated sites in the same molecule (e.g., see Stark and Boocock 1995).

The reduced XerC cleavage of *dif8* (and other sites that show resolution selectivity) on the linear suicide substrates used here, and on synthetic Holliday junction-containing substrates (L.K. Arciszewska and D.J. Sherratt, unpubl.) suggests that the accessory sequences and proteins are not only required for synapsis of two *dif8* sites, but may be necessary to alter the geometry of the core site/recombinase complex within the whole synaptic structure. We propose that this allows XerC to interact correctly with its binding site, so that strand exchange can be initiated. From in vitro recombination experiments on supercoiled substrates containing two directly repeated plasmid recombination sites, we know that XerC strand exchange precedes XerD exchange and that the synaptic complexes containing these sites must have a fixed local geometry (Colloms et al. 1996; S. Colloms, J. Bath, and D.J. Sherratt, unpubl.). However, as yet, we are not able to assay synapse formation and structure biochemically and therefore, cannot test directly the above hypothesis. Note that we do not believe recombination on linear suicide substrates requires syn-

apsis of two core site fragments (G. Blakely and D.J. Sherratt, unpubl.).

It seems likely that the inefficient cleavage of *dif8* by XerC is a consequence of the altered XerC interactions with the inner dyad (CATAA) portion of the left half-site when XerD is present. The alterations, which probably result from the changed geometry of the recombinase/core site complex, are indicated by reduced protection of the CATAA dyad sequence by XerC with both footprinting reagents and possibly an additional bend in *dif8* as suggested by the enhanced OP-Cu cleavages at the 3′ end of the central region adjacent to the right half-site. However, the footprinting data do not provide additional insight into the structural basis for and positions of all the bends in *dif6* and *dif8*.

In *dif6*, XerC and XerD together protect all of the central region from chemical attack by OP-Cu and also largely protect it from hydroxyl radical attack. The footprints shown here and earlier (Blakely et al. 1993) demonstrate that each recombinase spans the central region strand that they cleave (Fig. 4), which suggests that XerC and XerD may be in close contact with each other in this region. Such contacts may in part be responsible for the cooperative interactions between the two recombinases. In contrast, the *dif8* central region is sensitive to cleavage by both reagents when XerC and XerD are bound, demonstrating that specific domains of the recombinases have moved away from the central region DNA possibly to facilitate formation of cooperative protein/protein interactions. Although we have no firm evidence that indicates which part of each recombinase is in contact with the central region, we believe that one or both conserved arginines implicated in phosphodiester activation (Argos et al. 1986; Abremski and Hoess 1992; Chen et al. 1992) will interact with the CATAA dyad motif before XerC and XerD cleavage. Therefore, regions close to, or including, domain II may contact the central region. For XerD we have demonstrated directly an interaction between Arg247 and the phosphodiester that is cleaved (G. Blakely and D. Sherratt, unpubl.). This conserved domain II arginine, which is involved in phosphodiester activation, is present in regions of XerC and XerD that are predicted by computer analysis to have alpha helical secondary structure (G. Blakely and D. Sherratt, unpubl.). Such interaction of a helical XerC domain II with the major groove of the left half-site dyad could allow cleavage by OP-Cu and protection against hydroxyl radical cleavage. Similar findings were obtained with *Eco*RI; major groove interactions of a helical domain containing the catalytic site with the GAA recognition half-site gave protection against hydroxyl radical cleavage but allowed complete accessibility to OP-Cu (Kuwabara et al. 1986; McClarin et al. 1986).

The four- to fivefold difference in relative affinities of recombinase binding to *dif6* and *dif8* is not a dramatic change. Comparison of affinities for dimers of phage HK022 cI repressor bound at sites separated by variable spacing demonstrated that a 2-bp insertion led to a 1000-fold decrease in cooperativity (Mao et al. 1994). Lack of flexibility in the protein structure was used to explain this cooperativity loss. The relatively small reduction in cooperative interactions between XerC and XerD bound at *dif8* could suggest that one or both of the proteins contain a flexible region near the presumptive heterodimerization domain. Alternatively, distortion in the DNA induced by recombinase binding, rather than protein flexibility, could explain the relatively small loss in cooperativity. The differences in recombinase-induced bending are consistent with this idea, as is the gain in chemical sensitivity in the *dif8* central region and the left half-site. If XerC is "anchored" to the outer specificity region of its binding site (Fig. 1A) and the recombinase is fairly inflexible, then it may be pulled away from the dyad and central region of *dif8* DNA causing altered bending to maintain XerC/XerD contacts.

Our demonstration that differences in spacing between binding sites for two interacting proteins can give rise to biologically different outcomes is not unique. For example, the steroid receptor-binding sites can have variable spacing ranging from 1 to 5 bp. Identical binding sites with a 1-bp spacing give a retinoid X receptor-binding site, whereas a 4-bp spacing produces a T3 response element (Shulemovich et al. 1995). Similarly, it seems possible that topological filtering of the type that we believe is involved in resolution selectivity described here (discussed in Stark and Boocock 1995) is widely used to discriminate between arrays of distant sites containing bound proteins in complexes of fixed architecture and geometry. This may ensure that enhancers and their bound transcription factors activate appropriate genes at the correct time during differentiation and development (for review, see Corces 1995; Chi et al. 1995; Wijgerde et al. 1995).

Materials and methods

Strains and plasmids

All strains were derivatives of *E. coli* K12 AB1157 (Bachman 1972). The Xer$^+$ strain DS941 is AB1157 *recF lacI^q lacZ*ΔDM15; DS9009 is DS941 *xerD2*::Tn*10-9 xerCY17*; DS956 is DS941 *argR::fol*; DS957 is DS941 *pepA*::Tn*5* (Stirling et al. 1988; Colloms et al. 1990; Blakely et al. 1993; McCulloch et al. 1994). Construction of plasmid pMIN33 containing *dif6* has been described (Blakely et al. 1991). Synthetic oligonucleotides corresponding to the *dif8* sequence were annealed and ligated into the *Xba*I and *Sal*I restriction sites of pUC18 to give plasmid pGB300. Derivatives of core recombination sites with accessory sequences were constructed by digestion of the *cer* containing plasmid pKS492 (Stirling et al. 1988) with *Mlu*I followed by Klenow treatment and subsequent digestion with *Eco*RI; this 193-bp fragment was ligated to either pMIN33 or pGB300, which had been digested with *Xba*I, treated with Klenow, and then digested with *Eco*RI to give pGB305 and pGB306, respectively. Plasmids for circular permutation analysis were generated by ligating oligonucleotides containing *dif6* or *dif8* into *Xba*I and *Sal*I sites of pBEND2 (Kim et al. 1989). DNA sequences were confirmed using the dideoxy chain termination sequencing method.

DNA methods

Routine methods for DNA isolation and in vitro manipulation

were used (Sambrook et al. 1989). Oligonucleotides were labeled using T4 polynucleotide kinase with [γ-^{32}P]ATP. Restriction fragments for footprinting and pBEND2 analysis were labeled using the Klenow fragment of DNA polymerase with the appropriate [α-^{32}P]dNTP as described previously (Blakely and Sherratt 1994).

Oligonucleotides

Synthetic oligonucleotides (compare to *dif6* in Blakely et al. 1991) containing the *dif8* sequence were as follows: top strand, 5′-CTAGAATTGGTGCGCATAATTTGTATATTATGTTAAATCAG; and bottom strand, 5′-TCGACTGATTTAACATAATATACAAATTATGCGCACCAATT.

Gel retardation and proteins

Binding of XerC and XerD to DNA fragments was performed in buffer containing 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mg/ml of poly[d(I-C)] and 0.1 pmoles of labeled DNA for 10 min at 37°C before electrophoresis through 6% polyacrylamide in 1× TBE buffer at 4°C. Bend angles were determined from 8% polyacrylamide gels using the method of Thompson and Landy (1988). XerC and XerD were purified by fast protein liquid chromatography on Mono S columns as described previously (Blakely et al. 1993). Maltose-binding protein fusions were prepared as described in Arcizewska and Sherratt (1995). We have not determined what proportion of our recombinase preparations are active in binding or recombination. Apparent K_ds were determined as the protein concentration that bound 50% of DNA substrate.

In vitro cleavage assays

Suicide substrates were constructed by annealing two oligonucleotides, corresponding to left and right halves of top strand core sites flanked by pUC18 sequences, to an oligonucleotide corresponding to the bottom strand. Resulting double-stranded DNA thus contained a nick in the top strand of the core site central region. XerC and XerD (final concentration of 1 μM for each) were added to 0.1 pmole of labeled substrate in binding buffer (as above) and incubated at 37°C for 1 hr. Reactions were split in two; one portion was electrophoresed through 6% polyacrylamide under nondenaturing conditions in 1× TBE buffer, the other portion being treated with SDS and EDTA to give final concentrations of 0.1% and 1 mM, respectively. SDS treated samples were then electrophoresed through 6% polyacrylamide + 0.1% SDS in 1× TBE buffer containing 0.1% SDS.

In situ OP-Cu footprinting

End-labeling of top and bottom strands of *dif6* or *dif8* was performed using the 67-bp *Hin*dIII–*Kpn*I and the 73-bp *Eco*RI–*Sph*I fragments, respectively, from plasmids pMIN33 and pGB300. Scaled-up binding reactions containing 3.4 μg/ml of total protein were electrophoresed through 6% nondenaturing polyacrylamide, with retarded complexes detected by autoradiography at 4°C (Blakely et al. 1993). Excised gel fragments were immersed in 100 μl of 50 mM Tris-HCl (pH 8.0) before addition of 10 μl of the OP-Cu mix (2.0 mM 1,10-phenanthroline and 0.45 mM $CuSO_4$) and 10 μl of 58 mM mercaptoproprionic acid (Sigman et al. 1991); the mixture was gently vortexed and incubated at room temperature for 15 min. The reaction was terminated by addition of 20 μl of 2,9-dimethyl-1,10-phenanthroline. DNA fragments were eluted and precipitated before electrophoresis through 20% sequencing gels.

Hydroxyl radical footprinting

Binding reactions containing glycerol-free recombinases (~3 μg/ml) in 70 μl of 20 mM Tris-HCl (pH 7.5), 50 mM NaCl were incubated at 37°C for 10 min. Footprinting reactions, carried out at room temperature for 2 min, contained reagents at a final concentration of 120 μM [$(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$], 240 μM EDTA, 2 mM sodium ascorbate, and 0.03% H_2O_2 (Dixon et al. 1991). Reactions were stopped by adding 2 μl of 0.2 M EDTA and 20 μl of 50% glycerol before loading on a 6% nondenaturing polyacrylamide gel. Bound complexes were detected by autoradiography, excised from the gel, eluted and then precipitated. Samples were electrophoresed through 20% sequencing gels.

Quantitation

β-Particle emission from ^{32}P-labeled DNA in polyacrylamide gels was measured using a Molecular Dynamics PhosphorImager with ImageQuaNT software. Autoradiogram images were digitized using an Epson GT-6500 scanner and analysed using Image National Institutes of Health software (Dixon et al. 1991). DNA in agarose gels was stained with Sybr Green and quantified on a Molecular Dynamics FluorImager using ImageQuaNT software.

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